LESSON 28:
GENE MANIPULATION WITH AGROBACTERIUM TUMAEFACIENS

Introduction

Recombinant DNA technology, which has been used extensively with microbial systems, is also an important tool for the direct genetic manipulation of plants. There are a number of effective DNA-delivery systems and expression vectors that work with a range of plant cells. Furthermore, plant cells are totipotent, i.e. an entire plant can be regenerated from a single plant cell. Therefore, fertile plants can be produced with all cells carrying introduced gene (transgenic plants) from genetically engineered cells. If the transgenic plant produces viable seeds, the desired trait is passed on to successive generations.

What are the main objectives of developing transgenic plants?

There are three major reasos for developing transgenic plants.

1. The addition of a gene often improves the agricultural, horticultural or ornamental value of

   a crop plant.

2. Transgenic plants can act as living bioreactors for the inexpensive production of economically important proteins or metabolites.
3. Plant genetic transformation (transgenesis) provides a system to study the gene action during biological developmental processes.

Some of the genetically determined traits that can be introduced into plants by gene transfer methods are insecticidal activity, protection against virus infection, resistance to herbicides, altered flower colour, improved protein quality, and self-incompatibility. The transgenic plants produced with such characters are described in this chapter. It is important to note that gene/s for such characters are always integrated into plasmids. For higher plants, Ti plasmid of Agrobacterium tumefaciens is used, whether we use physical methods of direct gene transfer (next Lesson) or Agrobacterium mediated gene transfer system.

Crown gall disease of dicotyledonous species is caused by infection of the bacteria, Agrobacterium tumefaciens, and is a neoplastic growth. Crown gall is the most extensively studied disease and leads to the isolation of plasmid for genetic manipulations. Neoplastic transformation leads to an apparently stable alteration in cellular phenotype. A. tumefaciens utilizes opines produced by host (tumour) cells as carbon and nitrogen sources.

Ti Plasmid

Oncogenic strains of A. tumefaciens possess a large plasmid (90-150 x 106 daltons) known as the tumour inducing (Ti) plasmid. Transformation is associated with and accomplished by transfer of a stable, replicating portion of Ti plasmid DNA to the plant cell.

Although the precise mechanism of T-DNA (transferred DNA) transfer is not understood, molecular analysis of T-DNA integrated in host chromosomes has shown the presence of 25 nucleotides directly repeated sequences flanking the boundaries of the integration sites (left boundary and right boundary, LB, RB). Genes that are to be introduced in the plant cells must be inserted between these left and right border sequences, or just adjacent to one border of T-DNA.

Structure of Ti plasmid

Ti-plasmids have four distinct regions (Fig.1.)

A. T-DNA, main transfer DNA responsible for tumour formation

B. Responsible for replication

C. Responsible for conjugation

D. Responsible for virulence (vir region, vir-genes responsible for virulence; mutation in this region may lead to non-virulence). Important region required for transfer of T-DNA.

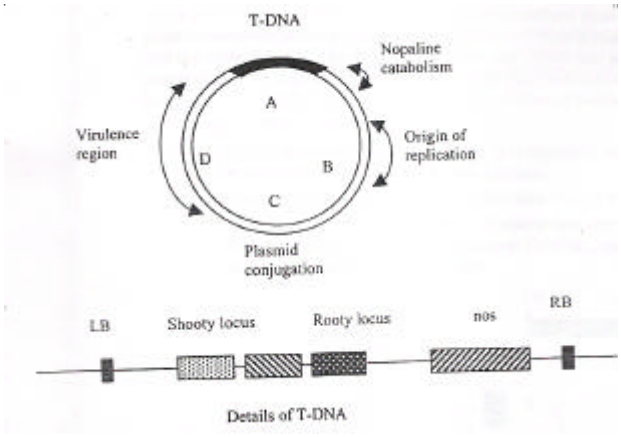

Fig. 1. Structure of Ti plasmid and T- DNA

Structure of T-DNA

T-DNA is transferred and integrated into host genome during the infection. Infection brings about physiological and morphological changes in the tissue due to expression of genes located on T.

These are:

Onc region - shooty, rooty genes, IAA and cytokinin production (Fig.1.)

OS region - opines - unusual amino acids, octopine and nopaline. Octopine synthase and nopaline synthase (nos) coded by T-DNA. So depending upon synthesis of amino acids plasmid is known as:

a. Octopine type - Ti plasmid, or

b. Nopaline type - Ti plasmid. These amino acids are used only by bacteria as source of carbon and nitrogen and these genes are located outside T-DNA.

Other important point of interest is 25bp flanking border sequences (Lb, Rb),

Vir region - If vir region and T -DNA are physically separated but present on two plasmids in the same bacteria, transfer of DNA takes place. This is an important property for vector. It consists of 36 kbp, and six operons: Vir- A, B, C, D, E & G. Except A&G others are polycystronic. Operons ABDG are for virulence. Operons C&E are for tumour formation.

Vir-A-chemoreceptor-senses presence of phenolics from wound-acetosyringone, B-hydroxy acetosyringone. Vir A transduces this information, most likely by a mechanism involving protein phosphorylation, to the product Vir-G. Vir-G then acts as transcriptional activator of itself and the other Vir loci. The products of Vir-C and Vir-D loci are involved in the generation and processing of the T-DNA copy. The products of the Vir-B and Vir-E loci are involved in forming most of the structural components that facilitate T-DNA movement.

T -DNA Transfer Process

It involves the following steps:

- Nicking between 3rd and 4th base of 25 bp repeats. Vir-D operon encodes for an endonuclease that causes nick formation.
- Initiation of DNA synthesis in 5'-3' direction.
- Involvement of bacterial genome - synthesis and secretion of glucose, cellulose, fibrils, and cell surface proteins. This is common physiological response in all soil bacteria and is involved with pathogenic characters.

The generation of the T-strand is the first step in the complex process of Agrobacteriummediated plant cell transformation. Following its formation, this DNA must traverse the bacterial cell membrane, the bacterial cell wall, the plant cell wall and plant cell and nuclear membranes. Once inside the nucleus, the T-DNA must finally integrate stably into the plant cell genome. During this entire transit process, the T-DNA strand also must avoid degradation by nuclease. The T-DNA exists as a DNA protein complex. The T-DNA complex protects it and mediates its travel. The final step in the genetic transfonnation of plant cell is integration of T-DNA copy, presumably T-strand, into plant cell DNA. It has been argued that the T -strand might be converted to a double stranded (ds) DNA prior to integration.

Thus, by placing foreign genes into T-DNA region of Ti - plasmid, it is possible to clone (make copies) the introduced genes with the multiplication of plasmid residing inside the bacteria (self replication of plasmid makes millions of copies) which is grown on a medium and with the multiplication of bacterial population, residing plasmid is also multiplied by this method. It is possible to exploit the natural ability of Agrobacterium to transfer new DNA into the plant genome.

Vectors Based on Ti and Ri Plasmids

The Ti or Ri- plasmid cannot be used directly. There are limitations for direct use of these plasmids. These are:

i. Large size of vector make it difficult to manipulate

ii. Absence of unique restriction enzymes sites and

iii. Tumour induction. Therefore, vectors are designed with useful characteristics. This involves - removal of tumour induction property or disarming the plasmid. This is achieved by replacing of tumour induction genes in T-DNA by selectable markers such as npt-II (kanamycin). Promoters and polyadenylation signal isolated from octopine and nopaline synthase genes were used for expression of selectable markers. As there is no excess production of plant hormones, whole plants transformed with such disarmed Agrobacterium strains can be produced and detected by the production of opines. When a selectable marker gene (kanamycin resistant) is introduced, transformed cells can be selected by their ability to grow on media containing the se1ective antibiotic. Untransformed cells will not survive on this medium.

Other promoters - onc – CaMV 35S, CaMV 19S isolated from cauliflower mosaic virus have also been used. Therefore, it is concluded that T-DNA and Vir genes are two essential components of a vector.

Cointegrative Vectors

Cointegrative vectors recombine, via DNA homology, with an intermediate cloning vector, which is used for manipulation and cloning of the gene in E. coli. Agrobacterium containing cointegrative vector and E. coli containing intermediate cloning vector are allowed to undergo conjugation, but the intermediate vector can not replicate in Agrobacterium so it has to transfer the marker genes as well as the DNA segment to the resident Ti plasmid (cointegrative vector) through recombination in the region of DNA homology. (Fig.2.)

Example of such vector - pGV3850 from nopaline type Ti plasmid, where almost all T-DNA has been replaced by pBR322, a small E. coli cloning vector (Bolivar and Rodriguez prepared and hence name - plasmid BR, followed by experiment number). The intermediate vector (pGV1103) based on pBR322 is conjugated into pGV3850 at the region of pBR322 homology.

Binary Vector

A significant advance that bypasses the problem of Ti-plasmid size was the discovery that the TDNA and the vir region could be separated on two different plasmids without loss of the T-DNA transfer capacity, i.e., they worked in a trans as well as a cis configuration. This discovery led to the development of binary T-DNA vectors that involve two plasmids. The small binary T-DNA plasmid has a wide host range that can replicate both in E. coli and Agrobacterium cells. The desired foreign gene is inserted into the binary T-DNA plasmid between the left and right border sequences (Fig.2A). A selectable plant marker gene is also inserted and (alongwith desired foreign gene) allow selection of transformed plant material. Several plant species have been transformed by this method.

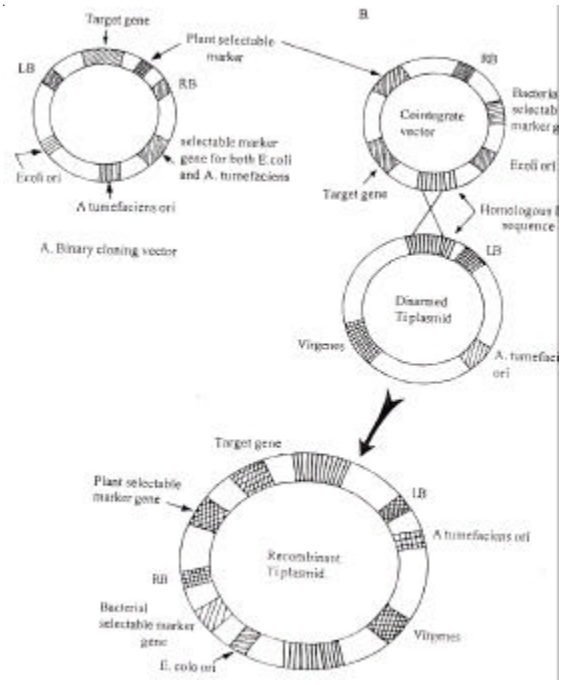

Fig. 2. Two Ti plasmid-derived cloning vector systems. A. the binary cloning vector which can replicate in E. coli and A. tumefaciens. B. the cointegrate vector carries only an E. coli origin of replication; it is fused with disarmed Ti plasmid (replicate in Agrobacterium) to develop recombinant Ti plasmid which has all the essential genes to replicate in different host cells, selectable marker genes and target DNA inserted between left and right border of T-DNA.

Transformation Technique

The critical information that made Agrobacterium mediated gene transfer systems possible came from the elegant work by Chilton et al. (1977) who showed that in crown gall disease a region of bacterial plasmid DNA (the T- DNA) is transferred to chromosomal DNA in the plant nucleus, stably maintained there, and expressed in the absence of the bacterium. The first transgenic Nicotiana tabacum plant was produced by Horsh and co-workers in 1984 using Agrobacterium.

Agrobacterium mediated gene transfer methods are being developed for a wide range of dicotyledonous plants and gymnosperm species. The gene tagging approach employs:

T-DNA containing either a reporter or strong transcriptional enhancer transforms a large number of cells. Normally bacteria are incubated with plant cells (few hours to few days). During that period T -DNA transfer takes place. The cells are then washed and treated with antibiotics to remove the bacteria. The cells are then cultured in the presence of the selectable agent, and transformed shoots are regenerated and characterised (Fig.3.).

Plasmids of Agrobacterium have been used as vectors for transfer of foreign DNA into a number of dicot species. However, seed legumes are still not amenable, exceptions Glycine max and monocotyledons (cereals) cannot be used, as Agrobacterium is host specific and does not infect monocots. But, with an exception of Asparagus (which has been transformed with this technique). There is no cambial activity and wound healing process in monocots, therefore, acetosyringone is not produced; this results in failure of vir genes to recognise by chemoreception.

Pre-requisites for agroinfection

1. Production of acetosyringone
2. Bacteria have access to actively dividing cells (DNA replication) such as meristems, fresh protoplasts, dedifferentiated tissues.
3. Regeneration in transformed tissues should be possible.

Explants for cocultivation- following materials can be used:

- Protoplasts
- Cell suspension cultures
- Callus \ thin cell layers - epidermis, tissue slice, organ section (leaf disc, section of roots, stem or flower)
- Wounded and inoculated whole plant.

Infection of plant cells with Agrobacterium

Agrobacterium carrying the plasmid vector in which the desired foreign gene sequences have been engineered, is directly inoculated at the wound site after removing the top leaves and apical meristem of the plants. Non-oncogenic vectors do not induce tumour formation and instead a wound callus proliferates in 3-4 weeks. The transformed callus tissue is then excised and cultured in the medium supplemented with an appropriate combination of growth regulators to regenerate transformed fertile plants. Other approaches followed are:

i. Co-cultivation with protoplasts: Freshly isolated protoplasts are cocultivated with Agrobacterium for a few days, washed, and then cultured in an antibiotic-containing medium (to eliminate bacteria). This is followed by transferring the regenerating protoplasts to suitable media enabling the development of transformed callus and shoots/plants (e.g., tobacco and potato).

ii. Leaf disc infection method: Surface-sterilised leaf discs of plants (Petunia, tomato, and tobacco) are inoculated with Agrobacterium tumefaciens strains having a modified tumour inducing plasmid and culture for two days. The leaf discs that develop infection are transferred to a selection medium enriched only with kanamycin. Horsch et al. (1985) reported the regeneration of transformed plants within 2-4 weeks from such cultures (Fig. 3). This appears to be a simple method that combines gene transfer, plant regeneration and effective selection for transformation in a single process. Shoot segments may also be tried in place of leaf discs. Deak et al. (1986) infected shoot segments of alfalfa with Agrobacterium and successfully induced somatic embryos on the transformed callus.

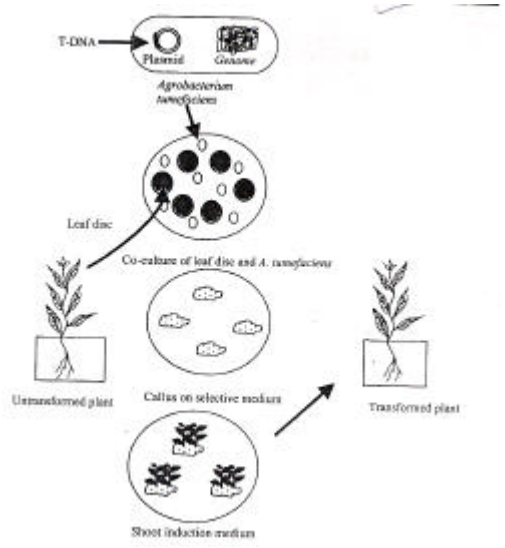

Fig. 3. Transformation procedure in laboratory

Selectable Markers

Why are selectable markers used?

Selectable markers are usually required for efficient recovery of transgenic cells and plants. After gene transfer, transformed cells are few in number compared to untransformed cells. It is not possible to separate transformed and non-transformed cells by any physical method. A selectable marker gene incorporated with the desired gene helps the growth of transformed cells on a nutrient medium containing corresponding selective agent. The availability of multiple selectable markers is useful in developing efficient transformation methods for diverse species as well as for the introduction of multiple novel traits (characters) through successful transformation and regeneration of transformed plants from such cells (Table1.).

Selective agents differ in their toxicity to different plant species. The different developmental states of the plant cells or tissues give different response to the selectable marker. The cells will react differently than whole plant or organ. Therefore, it is necessary to use correct concentration of selective agent for the transformed cells of a given species to select the cells and to inhibit the growth of untransfonned cells. This can be illustrated by following example. Transgenic plants of Lycopersicon esculentum, Brassica napus and Lactuca sativa can be selected on low kanamycin concentration (15-100 mg/l), while other plants like Beta vulgaris requires high kanamycin concentration (400 mg/l) as selection agent.

Table1. Selectable markers used in transformation.

Marker gene	*Enzyme encoded*	*Resistance against*
Antibiotics		
Npt-II	Neomycin phosphotransferase	Kanamycin, G418, neomycin
Npt or aph IV	Hygromycin phosphotransferase	Hygromycin
AadA	Aminoglycoside-3'-adenyl transferase	Streptomycin
Herbicides		
Bar	Phosphinothricine acetyltransferase	Phosphinothricin
Aro A	5-enolpyruvyl shikimate-3-phosphate synthase	Glyphosphate
Other modified als	Acetohydroxy acid synthase (or acetolactate synthase)	Chlorsulfuron imidazolanones
DHFR	Dihydrofolate reductase	Methotrexate

Herbicides are generally more toxic to plant cells than antibiotics. This is due to their specific mode of action in plant cells as well as their efficient uptake and translocation within the plant tissues. Herbicides and other highly toxic compounds may require delayed application in order to ensure that the transformed cells have produced sufficient quantities of enzyme responsible for the protection to such compound.

Callus and cell cultures are better systems than organized explants to achieve transformations and selection of transformed cells. Explants may give rise to organs from untransformed cells which may escape selective agent. This is not possible with isolated cells and only transformed cells will be able to grow on the selective agent.

Reporter Genes

In all transformation experiments, regeneration of non-transformed plants is also observed alongwith transformed plants. If these non-transformed plants can be detected at an early stage, a lot of time, labour and analyses can be saved. There are several methods to screen and identify the transformed plants, but usually these methods are laborious and time consuming, e.g. southern blotting technique to compare DNA sequences requires large amount of tissue (DNA) for comparison. The other methods use selective agent. In both these methods, prolonged growth and subcultures are involved before selection can be made. The use of reporter gene eliminates these drawbacks and transformed plants can be recognised easily.

What is a Reporter Gene?

A reporter gene is a coding unit whose product is easily assayed (such as GUS whose product. can easily be detected by histochemical assay). This gene may be connected to any promoter of interest so that expression of the gene can be used to assay promoter function. In fact, the reporter gene describes the transfer and expression of other promoter. Two genes that are now used for this purpose are those coding for B-glucoronidase (GUS) and luciferase (LUC). The coding regions of these non-plant genes have been fused to plant promoters and polyadenylation sequences, such that they give high level of expression in plant cells (Table 2.). The anthocyanin regulatory genes can serve as a unique reporter system in maize and some other cereals. Transformed cells accumulate reddish purple anthocyanin pigments. The anthocyanin genes are extremely sensitive marker used in Arabidopsis and Nicotiana transgenic plants.

Without selection agent, transformed plants are selected with the help of scoreable gene or reporter gene.

Table 2. Reporter genes, assay and identification method in transferred plants.

Reporter genes used for	Substrate and assay	Identification
Chloramphenicol acetyl transferase (CAT)	^{14}C cgkiranogebucik + acetyl Co-A, TLC separation	phenicol by autoradiography phenicol by autoradiography
B-glucoronidase (GUS)	Glucoronides (PNPG, X-GLUC, NAG, REG)	Fluorescence detection colorimetic, fluorimetric and histochemical
B-galactosidase (Lac Z)	β-glactoside (x-gal)	Colour of colony
Luciferase (LUC)	Decanal and $FMNH_2$ ATP + O_2 + luciferin	Bioluminiscence (exposure of x-ray ray (films)
Octapine synthase	Arginine pyruvate + NADH	Electrophoresis
Nopaline synthase	Arginine+ketoglutaric acid+NADH	—do—

Gene Expression and Integration

The primary objective of gene cloning for biotechnological applications is the expression of the cloned gene in a selected host organism. Unfortunately, the insertion of a gene into a cloning vector does not necessarily ensure that it will be expressed. Moreover, for commercial purposes, a high rate of production of the protein encoded by the cloned gene is required. To meet this demand, many expression vectors have been developed by manipulating a number of different genetic elements that control aspects of transcription, translation, protein stability, oxygen limitation and secretion from the host cell.

Following molecular features of gene expression are modulated:

1. the nature of the relevant transcriptional promoter and terminator sequences,
2. the strength of the ribosome binding site,
3. the number of copies of cloned gene and whether the gene is plasmid borne or integrated into the host genome,
4. the final cellular location of the synthesized foreign protein,
5. the efficiency of translation in the host organism, and
6. the intrinsic stability of the cloned gene protein within the host cell.

There is no single technique or method to achieve maximal expression of every cloned gene. Currently, although a wide range of both prokaryotic and eukaryotic organisms can express foreign genes, most commercially important protein products that are produced by recombinant DNA technology are synthesized in Escherichia coli. This is the most extensively studied organism in relation to its genetics, molecular biology, and physiology. Other host systems, such as Bacillus subtilis, yeast, and animal, plant and insect cells are also used to express certain cloned genes.

The production of a protein from a gene requires that the gene be properly transcribed, and the mRNA formed is translated. In prokaryotes, a promoter region is necessary for the initiation of transcription of the gene at the correct nucleotide site, and a terminator sequence at the end of gene is essential for stopping the transcription. Cloned genes often lack these signals. These signals must be in the correct locations for expression of a cloned gene in a prokaryotic host cell. It is also necessary to use a promoter that supports transcription at a high level (strong promoter) and that is compatible with the RNA polymerase of the host cell. But, continuous transcription of a cloned gene consumes all the energy of the host cell. Therefore, it is also necessary to use a promoter system whose activity can be regulated, e.g., by addition of a compound.The production of the protein is increased by increasing the numbers of copies of cloned gene.

This also enhances the stability of product. However, during scale-up of plasmid-based systems, plasmid-DNA insert contructs can be lost. This plasmid instability is undesirable for commercial systems. To overcome this problem, researchers have developed protocols, for integrating a cloned gene into a chromosomal site of the host organism. Under these conditions, gene is maintained stably as a part of the DNA of the host organism.

The introduction and expression of foreign DNA in a host organism often changes the metabolism of the organism and thereby impair its normal functioning. Therefore, several methods are developed for proper expression of the cloned gene and functioning of the host cell.

Conclusion

The Agrobacterium tumefaciens mediated gene transfer technology has enabled mobilization of genes from any organism or even synthetic DNA sequences into the genome of plant species (potentially). This capability can be and has been exploited to tailor plant genomes to suit various human needs. Many gene transfers of economic value like insect resistance, virus resistance etc. have been accomplished which are useful in crop improvement.

Questions

1. What are plasmids? Describe the plasmid mediated gene transfer process.
2. How does Agrbacterium tumefaciens help in mediating gene transfer? What are the methods by which it can be integrated into the host genome?
3. Write short notes on the following:
   a. Ti plasmid
   b. Agrobacterium
   c. Vector
   d. Binary vector
   e. T-DNA
   f. Vir gene
   g. T-DNA tansfer
   h. Selectable marker
   i. Reporter gene
   j. Gene expression
4. What are transgenic plants? Why is there a need to develop transgenic plants?

Note